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(54) Additive for use in feed for sows and feed for sows.

(57) The present invention relates to improvement of breeding efficiency of sows.

The present invention povides an additive for the feed of sows containing as an active ingredient a reduced form of folic acid in the form of 7,8-dihydrofolic acid, leucovorin, liver powder, disrupted cells or cell extract of a microorganism, etc.; a feed for sows added with such as additive; and a method of breeding sows in which sows are orally administered with such an additive or fed with such a feed.

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[Field of the Invention]

The present invention relates to an additive for use in feed for sows which contains as an active ingredient a reduced form of folic acid having an action of increasing the quantity of reduced form of folic acid in blood plasma of the sows and, as a result, of improving the breeding efficiency of the sows, and to a feed for sows added with such a feed additive.

[Prior Art]

Folic acid is a coenzyme participating in the synthesis of amino acids, such as methionine, serine and glutamic acid, and purine bases of nucleic acids constituting DNAs. It has been confirmed by epidemiological surveys on pregnant women and tests on pregnant guinea pigs that the demand for folic acid increases and its concentration in blood plasma decreases in mother's bodies during pregnancy [Pritchard J. A. et al., Am. J. Obst. Gynecol., Vol. 104, p. 388 (1969) and Habibzadeh H. C. et al., Br. J. Nutr., Vol. 55, p.23 (1986)].

It has also been confirmed with regard to pigs, the domestic animal, that the amount of reduced form of folic acids in blood plasma of sows decreases during pregnancy [Natsuhori et al., Final Program and Abstracts Book of the 10th International Symposium: "Chemistry and Biology of Pteridines and Flates," p.196 (1993)]. It has also been proved that breeding efficiency can be improved by administering folic acid (an oxidized form) to pregnant pigs by means of intramuscular injection [Matte J. J. et al., J. Anim. Sci., Vol. 67 p. 426 (1989) and Friendship R. M. et al., Can Vet. J., Vol. 32, p.564 (1991)], which shows the importance of administering folic acids to pregnant pigs (sows).

However, administering the folic acid to sows by intramolecular injection is practically troublesome, and it is highly preferred if the effect of improving breeding efficiency expresses when the folic acid are administered by means of addition to feed (oral administration). A number of investigations have already been made on the improvement of breeding efficiency based on oral administration. However, some consider the administration effective [Thaler R. C. et al., J. Anim. Sci., Vol. 67, p. 3,360 (1989), Lindemann M. D. et al., J. Anim. Sci., Vol. 67, p. 459 (1989) and Lindemann M. D. et al., J. Anim. Sci., Vol. 71, p. 239 (1991)], and others are skeptical about its effects [Easter R. A. et al., Nutrition Reports International, Vol. 28, p. 945 (1983) and Matte J. J. et al., Livestock Production Science, Vol. 33, p. 131 (1992)]. There is no clear conclusion on the effects of oral administration of an oxidized form of folic acid.

In general, folic acid is chemically synthesized. Chemically synthesized folic acid is of the oxidized form, and the oxidized form of folic acid per se does not function as a coenzyme. Usually, after being absorbed into the body, it is transferred by dihydrofolic acid dehydrogenase into 7,8-dihydrofolic acid, which is then enzymatically reduced to reduced forms of folic acid such as tetrahydrofolic acid (THF) or 5-methyltetrahydrofolic acid (5MF) to express the function as a coenzyme. Therefore, the effect of the administration of folic acids can be determined by measuring the amount of THF or 5MF in blood plasma. However, it is not possible to determine reduced forms of folic acid alone in a selective manner by the prior determination method since it is based on the radioligand technique, and hence it is not possible to obtain an accurate value of reduced forms of folic acid contained in blood plasma. In the present specification, reduced forms of folic acid may be referred to as "active-type folic acids" since they have physiological activities, and oxidized forms of folic acid may be referred to as "inactive-type folic acids" since they have no physiological activities.

For the purpose of analyzing the effects of administering folic acids to pigs, the HPLC-ECD method has been developed recently for determining the content of active-type folic acids in blood plasma by high performance liquid chromatography using an electrochemical detector, and, by using this method, investigations have been made on the extent of appearance of THF and 5MF in blood plasma at the time when folic acid (an oxidized form) is administered to pigs by intravenous injection, intramuscular injection or oral administration. To be more specific, 4 grown-up pigs having a body weight of around 25 kg were subjected according to the Latin square method to 4 factors: intravenous injection (1 mg/kg of body weight), intramuscular injection (1 mg/kg of body weight), small dosage oral administration (1 mg/kg of body weight) and large dosage oral administration (50 mg/kg of body weight), of folic acid (an oxidized form), and tests were performed. As a result, the concentration of THF and 5MF in blood plasma increased in cases of intravenous injection, intramuscular injection and large dosage oral administration. Accordingly, it is considered that the administered folic acid (an oxidized form) was absorbed and converted to active-type folic acids in liver, etc. On the other hand, THF and 5MF did not appear in blood plasma in cases of small dosage oral administration. With regard to the above, see Eiichi Kokue et al., "Abstract Book of the 113th Convention of Japan Veterinary Society," p. 112 (1992). In connection to rats, it has been known that the

concentration of reduced forms of folic acid increase rapidly even when an oxidized form of folic acid is administered in small dosage [Tsunematu K. et al., Cong. Anom., Vol. 30, p. 113 (1990)].

From the above, it is considered that pigs possess a capability of converting inactive-type folic acids to active-type folic acids, but their capability of absorbing inactive-type folic acids from the digestive tract is far less than that of rats. It has also been found that an extremely large quantity of inactive-type folic acids must be administered to pigs in order to increase the value of active-type folic acids in blood plasma by oral administration of inactive-type folic acids.

[Problems to be Solved by the Invention]

The present invention has been made in view of the above prior art. Its objects are to provide an additive for use in feed for sows which is capable of increasing the concentration of reduced forms of folic acid in blood plasma and, as a result, of improving breeding efficiency, and to provide a feed for sows for improving breeding efficiency which is added with such an additive.

[Means Taken to Solve the Problems]

For the purpose of solving the above problems, the present inventors have conducted intensive investigations. As a result, the inventors have found that the concentration of reduced forms of folic acid in blood plasma of pigs can be increased by adding a reduced form of folic acid to feed and administering it orally to sows, and have completed the present invention on the basis of the finding.

Accordingly, the present invention is concerned with the improvement of breeding efficiency of sows through oral administration of a reduced form of folic acid. The invention will be explained hereinbelow in detail.

In the first place, the present invention is concerned with an additive for use in feed for sows which is characterized in that a reduced form of folic acid is contained as an active ingredient for improving the efficiency of breeding.

As is well known, a plurality of embryos (multiple embryos) are usually generated through fertilization in the uterus of sows. However, all the multiple embryos so generated are not always delivered safely. Of course, it is highly desirable from the viewpoint of the management of pig breeding that all the multiple embryos once generated can be safely delivered. In the present invention, the expression "breeding efficiency of sows improves" means that the rate of safely delivered embryos to the total number of embryos generated increases when the sows are orally administered with the feed additive according to the present invention or when they are fed with the feed according to the invention, in comparison with cases where they are not so administered or not so fed.

In the present invention, the term "a reduced form of folic acid" means not only a reduced form of folic acid in its narrow sense [pteroyl(mono)glutamic acid], but also a reduced form of other various folic acids (folic acids in a wider sense). It should be noted that the definition of a reduced form of folic acid include such reduced forms of folic acid which show the physiological functions similar to the reduced form of folic acid in its narrow sense.

Accordingly, in the present invention, reduced forms of folic acid include 7,8-dihydrofolic acid (H_2 folic acid) in which the pteridine ring of folic acid is reduced; H_4 folic acids, such as 5,6,7,8-tetrahydrofolic acid (H_4 folic acid), 5-formyl-H_4-folic acid, e.g. leucovorin [L-(-)-5-formyl-5,6,7,8-tetrahydrofolic acid], 5,10-methylene-H_4-folic acid, 5-methyl-H_4-folic acid, 10-formyl-H_4-folic acid, 5-methyl-H_4-folic acid, 5-formimino-H_4-folic acid, etc.; and derivatives, such as poly-gamma-glutamic acid derivatives of each H_4 folic acid (known as storage-forms of folic acid in the liver). It is a matter of course that in the present invention the reduced form of folic acid can also be in the form of liver powders and disrupted cells or cell extract of a microorganism containing a reduced form of folic acid.

By the way, liver is an organ in which metabolism of various vitamins is performed, and it has been known that active-type folic acids are contained in the liver in relatively high contents. However, no cases have been known wherein liver powders prepared, e.g., through freeze-drying and pulverization of livers of pigs or cows are orally administered to sows to improve their breeding efficiency. It is a novel knowledge by the present inventors that the concentration of THF and 5MF in blood plasma of pigs can be increased by incorporating liver powders into feed.

Regarding disrupted cells or cell extract of a microorganism, yeasts, such as Torula yeast, have been used as a source of vitamins for feed. However, vitamins contained in their cells are absorbed only poorly since they are usually used without disruption of the cell walls.

In view of the above, the present inventors have conducted intensive investigations and have found that the amount of THF and 5MF in blood plasma of pigs can be increased by orally administering disrupted cells or cell extract of a microorganism, namely, products prepared by subjecting the cells of a microorganism to a mechanical disruption treatment, to an enzymatic digestion treatment, or to autolysis, etc., so as to make the reduced forms of folic acid into a readily absorbable condition. Accordingly, the breeding efficiency can be improved by administering the product to pregnant sows.

Any microorganisms can be used as a raw material for producing the disrupted cells or cell extract, provided that the products (mechanically disrupted cells or enzymatically disrupted cells) have a high content of reduced forms of folic acid. Specific examples include bacteria, such as Corynebacterium glutamicum (former name: Brevibacterium lactofermentum) (ATCC 13,869, etc.), Corynebacterium ammoniagenes (former name: Brevibacterium ammoniagenes) (ATCC 6,871, etc.), Brevibacterium flavum (ATCC 13,826, etc.), Corynebacterium glutamicum (ATCC 13,032, ATCC 13,060, etc.), Bacillus subtilis (ATCC 13,952, IFO 3,009, IFO 13,169, etc.), Lactococcus lactis subsp. cremoris (ATCC 19,257, etc.), and the like; yeasts, such as Saccharomyces cerevisiae, (IFO 2,044, IFO 2,375, etc.), Candida utilis (former name: Torulopsis utilis) (ATCC 9,226, etc.), and the like; and fungi, such as Aspergillus oryzae (IFO 30,104, etc.), Aspergillus niger (IFO 4,414, etc.), and the like.

Any culturing media can be used for the culturing of these microorganisms, provided that nutrients assimilable to the microorganisms are contained therein. For example, there can be used ordinary media added appropriately with carbon sources, such as carbohydrates, such as glucose, sucrose, etc., alcohols, such as ethanol, glycerol, etc., organic acids, such as acetic acid, propionic acid, etc., soybean oils, and mixtures of these; nitrogen-containing organic or inorganic nutrients, such as yeast extract, peptone, meat extract, corn steep liquor, ammonium sulfate, ammonia, etc.; inorganic nutrients, such as phosphates, magnesium, iron, manganese, potassium, etc.; and vitamins, such as biotin, thiamine, etc.

For the culturing, conditions usually employed for the culturing of these microorganisms can be used without any modifications. For example, the microorganisms can be cultured in a nutrient medium at 20 to 40 °C at a pH in the range of 4.0 to 9.5 for a period of 20 hours to 5 days.

The amount of reduced forms of folic acid produced in the cells of a microorganism obtained by the culturing may increase by adding p-aminobenzoic acid, an oxidized form of folic acid, and/or a nucleic acid in the culture medium. The nucleic acid used contains guanosine, inosine, xanthine, 5'-guanilic acid, 5'-inosinic acid, 5'-xanthylic acid, guanosine-5'-diphophate, and guanosine-5'-triphosphate. These additives can be added in an amount where the amount of reduced forms of folic acid produced in the cells can be increased, in comparison with cases where the additives are not added; for example, in an amount of 1 mg/liter to 1 g/liter, preferably 10 to 100 mg/liter. If the amount added is too small, no effects will be attained, whereas if it is too much, the growth of the microorganism may be inhibited.

The cells thus obtained by the culturing are subjected to disruption or extraction treatment after being separated from the culture liquid by an appropriate method. But the culture can be subjected to the disruption or extraction treatment directly or after concentration, when the ingredients of the culture medium may be orally administered to the sows and if they do not affect the performance of the disruption treatment. Furthermore, the cells to be subjected to the disruption or extraction treatment can be live cells or killed cells.

There is no particular restriction on the method of disruption of the cells. For example, the disruption can be performed by a hitherto known mechanical method, as well as by a method utilizing enzymes. Mechanical methods per se can be carried out in accordance with prior ones. For example, the cells of a microorganism can be disrupted with glass beads by using "Beads Beater" (manufactured by Biospec Co.), or the disruption of the cells can be performed with pressure, or can be carried out by using an ultrasonic disrupter. Also, in the case where the cells of a microorganism are disrupted by an enzyme, the method per se can be carried out in accordance with a prior method. For example, after the cultured cells have been subjected to a heat sterilization treatment as they are, a cell wall digesting enzyme is added thereto, to decompose the cell walls of the microorganism. For this decomposition, any enzyme can be used, provided that it is capable of digesting and disrupting the cell walls. Well-known enzymes such as lysozymes, proteases, zymolyases, and the like, are typical examples of the enzymes having such a capability. The enzymatic treatment can be carried out in accordance with known conditions.

There is also no particular restriction on the method of extraction of the cells. For example, the extraction can be performed by autolysis or by heating the cells in hot water at a temperature range between 90 °C and 120 °C.

The thus prepared disrupted cells or cell extract can be orally administered either as they are, or in an appropriate concentrated or dried form, or in the form of a mixture added with appropriate additives. Since almost folic acids are not present in cell walls, it is possible to remove the remaining fragments of cell walls

from the disrupted cells. It is a matter of course that among possible forms of oral administration is included the one where the disrupted cells or cell extract is added to a feed and sows are fed with the feed.

It is also a matter of course that the various reduced forms of folic acid can be used individually, or two or more of them can be used in combination.

Taking into consideration the convenience of use, the feed additive for sows according to the present invention can be distributed in an appropriate dosage form, including concentrates, dried powders, granules, etc., with or without addition of appropriate carriers or the like.

In the second place, the present invention is concerned with a feed for sows which is characterized in that the feed additive for sows of the present invention is added thereto.

There is no particular difficulty on the production of such a feed for sows. It can be produced in accordance with a method known for the production of formula feeds, except that the feed additive according to the present invention is added thereto.

Explanation will then be given on a point to be considered with regard to the addition of the feed additive for sows of the present invention. It is the amount to be added. The feed additive for sows of the present invention is to be added in an amount where the effects of the addition appear. For example, it is added in an amount which gives an intake of 0.1 to 100 μg (as reduced form of folic acid) per kg of body weight of a sow per day. If the amount added is too small, no effects will be attained. The effects of the addition will not increase even if it is added in an amount greater than the above range and hence it will be useless.

In the third place, the present invention is concerned with a method of improving the breeding efficiency of sows which is characterized in that the feed additive for sows according to the present invention is orally administered to the sows, or the sows are fed with a feed according to the present invention.

There is no particular difficulty on such a method of improving the breeding efficiency. All the conditions on the breeding can be in accordance with hitherto known prior methods, except that the amount of a reduced form of folic acid taken is 0.1 to 100 μg, per kg of body weight of sows.

In the method of breeding of present invention, the duration of oral administration or feeding is as follows: Since the purpose of administering the reduced form of folic acid is to bring safely to birth multiple embryos generated by fertilization in the interior of the womb of a sow, as many as possible, if possible, all of them, the oral intake of reduced form of folic acid is started at a time between about two months before mating (fertilization) and just after mating, and the intake is continued until the multiple embryos or fetuses are confirmed to have grown up to a safe birth step (for example, two months after mating), or up to their birth for the sake of safety.

[EXAMPLES]

The present invention will further be explained by examples.

Example 1 (Effects of Administration of Leucovorin)

The tests were performed by using two Gettingen mini-pigs of the same litter (0.7 years old pigs having a body weight of 15 kg; one for the test and one for comparison).

A 0.75% water suspension of leucovorin (produced by Sigma Co.) was orally force-fed (directly injected into the interior of the stomach through a fine flexible tube) to the test animal which had been fasted from the day before, in an amount of 50 mg per kg of body weight. For the purpose of comparison, the force-feeding was performed in exactly the same manner, except that folic acid (an oxidized form; produced by Kongo Kagaku Co.) was used in place of leucovorin.

Blood samples were collected 1, 3, 6, 9 and 24 hours after the administration of the test substances, and two kinds of active-type folic acids, namely tetrahydrofolic acid (THF) and 5-methyltetrahydrofolic acid (5MF) contained in the blood plasmas of the pigs were quantitatively analyzed. As a control, blood was also collected just before the administration of the test substances, and the blood was subjected to the same quantitative analysis (control).

Details of the quantitative analysis were as follows. To 0.2 ml of a collected blood sample was added 0.2 ml of 0.5M perchloric acid, and the resulting mixture was subjected to centrifugation of 5,000 g x 2 min, to remove proteins. One hundred (100) μl of the supernatant obtained was subjected to high performance liquid chromatography. The analysis was performed using a column of "Phenyl-bonded phase 4.6 mm ϕ x 150 mm" (produced by Irica Co.). For the mobile phase, a mixture of 97.5:2.5 (v/v) of 20 mM potassium acetate buffer (pH 3.6) and acetonitrile was used, and the flow rate was 0.8 ml/min. For the detection,

"Electrochemical Detector Type E-502" (produced by Irica Co.) was used, and the determination was performed at an applied voltage of -300 mV.

Results of the determination are shown in the following Table 1.

[Table 1]

Time Lapsed (h)	Test		Comparison	
	THF (*)	5MF (*)	THF (*)	5MF (*)
0 (control)	8.2	6.0	14.6	7.6
1	51.5	12.1	14.6	4.5
3	52.1	20.6	12.0	10.5
6	55.7	27.6	12.3	7.1
9	49.2	31.0	12.4	7.9
24	30.2	20.6	8.0	6.3

*: unit: ng/ml

As would be apparent from Table 1, the concentration of THF in the blood plasma increased immediately after the administration of leucovorin, and then the concentration of 5MF increased. The behavior of the two active-type folic acids in blood is well understandable since it has been known that leucovorin changes to THF and then to 5MF in the metabolism in organisms. The fact that the concentration of THF in blood plasma increased within 1 hour indicates that the absorption of leucovorin into the body proceeds quite smoothly. On the other hand, in oral administration of folic acid (inactive type) carried out for comparison, no changes were observed in the value of folic acids in blood plasma.

Example 2 (Effects of Administration of 7,8-Dihydrofolic Acid)

The tests were performed by using two Gettingen mini-pigs of the same litter (2 years old pigs having a body weight of about 20 kg).

7,8-Dihydrofolic acid (produced by Sigma Co.) in 0.2 % sodium ascorbate solution was orally force-fed to the test animals which had been fasted for 24 hours, in an amount of 1 mg or 0.2 mg per kg of body weight. Blood samples were collected after the administration, and active-type folic acids contained in the blood plasmas were quantitatively analyzed in the same manner as in Example 1.

The change of the concentrations of THF and 5MF in the blood plasmas of the two pigs is shown in the following Table 2.

[Table 2]

Time Lapsed (hr)	1 mg/kg		0.2 mg/kg	
	THF (*)	5MF (*)	THF (*)	5MF(*)
0 (control)	6.75	4.44	7.22	4.74
1	17.52	4.45	14.51	5.01
3	20.94	2.47	15.09	1.94
6	14.51	1.89	15.32	1.42
9	12.49	1.88	17.46	1.88
24	6.49	2.39	14.39	3.37

*: unit: ng/ml

As is understood from Table 2, the administration of 7,8-dihydrofolic acid in both the amounts led to remarkable increase of THF concentration, although 5MF concentration decreased to some extent.

Example 3 (Effects of Administration of Liver Powders)

The test of was repeated exactly in the same manner as in Example 1, including the test animals, except that a 50% water suspension of powders of pig liver was force-fed in an amount of 5 g as liver powders (which contained oxidized forms and reduced forms of folic acid in an amount of 0.08 mg in total) instead of the water suspension of leucovorin. The results of the comparison test in Example 1 are also utilized here.

Results are shown in Table 3.

[Table 3]

Time Lapsed (h)	Test		Comparison	
	THF (*)	5MF (*)	THF (*)	5MF (*)
0 (control)	11.9	5.5	14.6	7.6
1	24.5	28.3	14.6	4.5
3	26.4	22.7	12.0	10.5
6	24.4	14.8	12.3	7.1
9	24.6	13.3	12.4	7.9
24	14.8	11.8	8.0	6.3

*: unit: ng/ml

As would be apparent from Table 3, in the test of liver powders, the concentration of THF increased quickly and the concentration of 5MF also increased. This is presumably because a large quantity of 5MF is contained in liver powders. On the other hand, in the comparison, as is mentioned in Example 1, no increase was observed in the concentration of 5MF and THF in blood plasma. It would be understood that the absorption of folic acids into body is extremely good, considering the fact that the content of folic acids in 5 g of liver powders is 0.08 g (including inactive-type folic acids) is taken into consideration, .

Example 4 (Preparation of Disrupted Cells of Microorganisms)

(a) Cultivation of Microorganisms

Into 500 ml flasks was poured 50 ml each of a culture medium having the composition shown in Table 4 set forth below. After sterilization by heating, one platinum loopful of cells of each of the microorganisms shown in Table 5 ---the bacteria used were previously cultured on a bouillon agar medium at 30 °C for 24 hours, and the yeasts and molds used were previously cultured on a malt extract agar medium at 30 °C for 48 to 72 hours --- were inoculated in the medium and cultured with shaking at 30 °C for 24 to 78 hours. After the culturing, cells were collected by centrifugation.

[Table 4]

Components	Concentraton
Glucose	2.0 g/dl
Yeast Extract	1.0 g/dl
Polypeptone	1.0 g/dl
$(NH_4)_2SO_4$	0.5 g/dl
K_2HPO_4	0.3 g/dl
KH_2PO_4	0.1 g/dl
$MgSO_4.7H_2O$	0.05 g/dl
$FeSO_4.7H_2O$	0.001 g/dl
$MnSO_4.4H_2O$	0.001 g/dl
pH 7.0	

[Table 5]

	Microorganisms	Contents of Folic acids (mg/100 g)	
		Before Disruption	After Disruption
Bacteria	Corynebacterium glutamicum ATCC 13869	2.0	12.0
	Corynebacterium ammoniagenes ATCC 6871	1.3	8.9
	Brevibacterium flavum ATCC 13826	2.8	14.2
	Corynebacterium glutamicum ATCC 13032	1.5	10.8
	Corynebacterium glutamicum ATCC 13060	3.5	9.0
	Corynebacterium glutamicum ATCC 13952	1.6	5.6
	Bacillus subtilis IFO 3009	0.9	4.7
	Bacillus subtilis IFO 13169	2.8	8.3
	Lactococcus lactis subsp. cremoris ATCC 19257	2.1	7.8
Yeasts	Saccharomyces serevisiae IFO 2044	0.3	3.8
	Saccharomyces cerevisiae IFO 2375	0.7	4.0
	Candida utilis ATCC 9226	0.1	2.9
Molds	Aspergillus oryzae IFO 30104	1.1	5.8
	Aspergillus niger IFO 4414	1.6	7.6

(b) Preparation of Disrupted Cells of Microorganisms

The microorganisms so collected were suspended into physiological saline of a volume equal to the culture medium and then subjected to a heat treatment (sterilization) at 100 • C for 10 minutes, and the cells were again collected by centrifugation. The cells were suspended into a 25 mM phosphate buffer (pH 7.0) at a concentration of 10% by wet weight.

With regard to bacteria, 0.1% by weight of egg-white lysozyme (produced by Sigma Co.) and 0.2% by weight of papain (produced by Amano Pharmaceutical Co.) were added to the thus prepared suspensions of the cells, and the cell walls were digested and disrupted by maintaining the mixture at 37 • C for 12 hours,

to obtain a disrupted cell liquid. With regard to yeasts, 0.2% by weight of "Zymolyase 20T," a yeast cell wall digesting enzyme (produced by Seikagaku Kogyo K.K.), was added, and the cell walls were digested and disrupted by also maintaining the mixture at 37°C for 12 hours, to obtain a disrupted cell liquid. With regard to molds, the suspensions of cells were added with the same quantity (by volume) of glass beads of 0.75 mmϕ, and the mixture was subjected 5 times to the cell disruption treatment of 1 minute by using "Beads Beater" (produced by Biospec Co.) and then to decantation, to obtain a supernatant by removing glass beads.

Each of the thus obtained disrupted cell liquids and supernatant fluids of bacteria, yeasts and molds was dried by freeze-drying to obtain powders (one of the distribution form of the additive for the feed of sows according to the present invention).

(c) Determination of the Content of Folic Acids

The amount of folic acids contained per 100 g of the thus obtained dried powders was determined by means of bioassay using Enterococcus hirae ATCC 8043. Results obtained are also shown in Table 5. In this bioassay, both the active-type folic acids (reduced forms) and inactive-type folic acids (oxidized forms) are determined at the same time to give their total amount. However, the value so determined may be regarded to be mostly based on active-type since the folic acids contained in the dried powders are derived from microorganisms.

Example 5 (Preparation of Mechanically Disrupted Cells of Microorganisms)

In a similar manner as in Example 3, Corynebacterium glutamicum ATCC 13869 and Corynebacterium glutamicum ATCC 13060 were cultured, and their cells were collected and suspended into 20 mM phosphate buffer (pH 7.0) at 10% by weight, to prepare cell suspensions.

The cell suspensions were admixed with the same quantity of glass beads of 0.1 mmϕ, and the cells were completely disrupted by repeating 10 times disruption treatment of 1 minute by using "Beads Beater." Thereafter, resulting products were subjected to centrifugal separation, to separate cytoplasm fractions into centrifugal supernatants and the cell wall fractions into centrifugal residues.

Each of the fractions were dried by freeze-drying, and the amount of folic acids present in 100 g of dried products was determined by means of bioassay as in Example 4. Results obtained are shown in Table 6.

[Table 6]

Bacteria	Contents of Folic Acids (mg/100g)	
	Cytoplasm Fraction	Cell Wall Fraction
Corynebacterium glutamicum ATCC 13869	13.6	0.006
Corynebacterium glutamicum ATCC 13060	11.2	0.008

It would be understood from Table 6 that folic acids are present in the cytoplasm fractions.

Example 6 (Cultivation with Addition of Folic Acid Precursors)

In a similar manner as in Example 4, digested products (disrupted products) of cells were prepared by enzymatic treatment of the cells using Corynebacterium glutamicum ATCC 13869 or Corynebacterium glutamicum ATCC 13060.

The cells used above were obtained by culturing the above bacteria with addition of 100 mg/liter of p-aminobenzoic acid, 10 mg/liter of folic acid (an oxidized form) or 100 mg/liter of guanosine.

The content of folic acids contained in 100 g of dried powders is as shown in Table 7.

[Table 7]

Bacteria	Content of Folic Acids (mg/100 g)							
	Added with p-Aminobenzoic Acid				Added with Folic Acid			
	Disruption treatment				Disruption treatment			
	before		after		before		after	
	Added with Guanosine				Added with Guanosine			
	Disruption treatment				Disruption treatment			
	before	after	before	after	before	after	before	after
Corynebacterium glutamicum ATCC 13869	4.2	23.0	3.4	21.9	3.0	18.5	3.4	18.5
Corynebacterium glutamicum ATCC 13060	3.6	22.4	2.9	18.7	3.8	15.7	3.8	15.7

Example 7 (Effects of Administration of Disrupted Cells of Microorganisms)

The effects of administering (1) the enzymatic digestion product of Corynebacterium glutamicum prepared in Example 4, (2) the enzymatic digestion product of Saccharomyces cerevisiae IFO 2044

prepared in Example 4, and (3) enzymatic digestion product of Corynebacterium glutamicum ATCC 13869 (cells cultured with addition of p-aminobenzoic acid to the medium) were tested in a similar manner as in Example 1.

As test animals were used 4 Gettingen mini-pigs of the same litter (1 year old; body weight, 30 kg). The enzymatic digestion products were used in an amount of 50 mg as the dried product, per 1 kg of body weight. For the purpose of comparison, the same test was carried out by using 50 mg of an inactive-type folic acid (produced by Kongo Kagaku K.K.), per 1 kg of body weight (comparison).

Results obtained are shown in Table 8 of the following.

[Table 8]

Time Lapsed (h)	Test						Comparison	
	Enzymatically Digested Product						Inactive-type Folic Acid	
	(1)		(2)		(3)			
	THF	5MF	THF	5MF	THF	5MF	THF	5MF
0 (control)	30.9	2.4	18.7	1.8	28.7	4.2	19.8	4.3
1	36.4	2.4	19.9	2.0	38.7	5.7	19.1	3.7
3	34.7	2.6	24.7	2.5	41.3	3.8	19.9	3.4
6	28.0	2.6	26.2	2.8	37.5	2.8	19.7	2.9
9	31.4	2.0	24.2	2.3	33.4	1.6	15.8	2.6
24	27.2	2.9	20.4	3.1	28.7	2.7	15.1	3.7

In the table, the amounts of THF and 5MF are shown by ng/ml.

The followings could be understood from Table 8. An increase in the value of THF in blood plasma was observed in each of the cases of the enzymatic digestion products (1), (2) and (3), in comparison with those of control (before administration). In the cases of (1) and (2), the concentration of THF speedily increased after the administration. In the case of (2), the rate of increase in the concentration of THF was slow in comparison with (1) and (3). With regard to the extent of increase in the concentration of THF, (3) is higher than (1) and hence is more effective. An inactive-type folic acid (comparison) has no effect of increasing the concentration of THF and 5MF in blood plasma.

Example 8 (Field Test)

There were used 60 (40 for the tests and 20 for the comparison) sows (age, 1.5 to 4 years old; body weight, 150 to 200 kg) of LW species (a hybrid formed by cross-breeding Landrace species with Large Yorkshire species). The disrupted cells of Corynebacterium glutamicum ATCC 13869 (dried product of enzymatically digested cells) prepared in Example 4 and the disrupted cells of the same strain (dried product of the cytoplasm fraction) prepared in Example 5 were used as active-type folic acid-containing products.

Starting 2 months before mating, 20 sows were continuously fed with a feed added with the folic acid-containing disrupted product prepared in Example 4 in an amount of 300 mg per day per sow. After 60 days from the mating, the content of THF and 5MF in blood plasma was determined as in Example 1 (Test I).

A similar test was conducted on the disrupted cells prepared in Example 5 (Test II). For the purpose of comparison, a similar test was carried out without adding any disrupted cells (Comparison).

Results are shown in Table 9. As would be understood from the table, the content of THF and that of 5MF become higher when administered with the folic acid-containing products (Tests I and II) as compared with Comparison.

[Table 9]

Test I				Test II				Comparison			
Sow No.	THF	5MF	Total	Sow No.	THM	5MF	Total	Sow No.	THM	5MF	Total
1	7.7	0.7	8.4	21	4.5	1.1	5.6	41	2.3	0.0	2.3
2	10.1	0.9	11.0	22	3.0	1.6	4.6	42	3.0	0.0	3.0
3	5.8	1.4	7.2	23	6.8	1.2	8.0	43	2.4	0.5	2.9
4	2.3	0.0	2.3	24	2.9	0.8	3.7	44	2.9	1.2	4.1
5	3.2	0.4	3.6	25	5.0	1.6	6.6	45	1.8	0.4	2.2
6	3.4	0.0	3.4	26	11.2	1.2	13.4	46	4.6	0.6	5.2
7	4.6	0.4	5.0	27	4.4	0.6	5.0	47	3.2	0.8	4.0
8	3.0	1.5	4.5	28	5.2	0.8	6.0	48	2.4	0.0	2.4
9	3.6	0.0	3.6	29	3.2	0.4	3.6	49	2.0	0.0	2.0
10	5.0	0.6	5.6	30	3.0	0.0	3.0	50	4.2	1.4	5.6
11	4.5	1.1	5.6	31	8.7	1.0	9.7	51	3.0	0.8	3.8
12	4.3	0.4	4.7	32	6.6	0.8	7.4	52	2.2	0.0	2.2
13	5.0	1.6	6.8	33	4.3	0.6	4.9	53	1.8	0.0	1.8

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Test I				Test II				Comparison			
Sow No.	THF	5MF	Total	Sow No.	THM	5MF	Total	Sow No.	THM	5MF	Total
14	6.1	1.2	7.3	34	3.2	0.8	4.0	54	1.8	0.0	1.8
15	4.8	0.6	5.4	35	5.0	1.2	6.2	55	3.0	1.4	4.4
16	5.8	1.4	7.2	36	3.8	1.0	4.8	56	2.4	0.8	3.2
17	5.0	1.5	6.5	37	2.6	0.0	2.6	57	3.0	0.0	3.0
18	2.3	0.8	3.1	38	4.2	0.4	4.6	58	2.8	0.6	3.4
19	4.5	0.8	5.3	39	3.6	1.0	4.6	59	3.8	1.0	4.8
20	2.5	0.9	3.4	40	3.4	0.6	4.0	60	2.0	0.6	2.6

In each of the tests, the administration of folic acids in the form of the disrupted cells of a microorganism was continued up to delivery.

All the sows delivered around 114 days after mating. The results of delivery were as follows: In the test administered with the enzymatically digested cells (Test I), 11.6 baby pigs were delivered in average, and in the test administered with the dried product of cytoplasmic fraction (Test II), 11.8 baby pigs were delivered

in average. On the other hand, 10.8 baby pigs were delivered in average in the case of comparison. The above results prove that the values of THF and 5MF in blood plasma of sows increase by administering the enzymatically digested cells and the dried products of cytoplasmic fractions (present invention), and hence the results of breeding can be improved.

[Merits of the Invention]

In accordance with the present invention, the concentration of active-type folic acids contained in blood plasma of sows can be increased through oral administration of a reduced form of folic acid and, as a result, the efficiency of breeding can be readily improved. This would contribute to the management of pig breeding to a great extent.

Claims

1. An additive for use in feed for sows comprising as an active ingredient for improving the efficiency of breeding a reduced form of folic acid or an active derivative thereof.

2. An additive for use in feed for sows according to Claim 1, wherein said reduced form of folic acid is in the form of 7,8-dihydrofolic acid, leucovorin, liver powders, and/or disrupted cells or cell extract of a microorganism.

3. An additive for use in feed for sows according to Claim 2, wherein said disrupted cells or cell extract of a microorganism is obtained from cells cultivated in a medium added with p-aminobenzoic acid, an oxidized form of folic acid, and/or a nucleic acid.

4. A feed for sows comprising a feed additive according to any of Claims 1 to 3.

5. A method of improving the efficiency of breeding of sows which is characterized in that a feed additive according to any of Claims 1 to 3 is orally administered to the sows, or the sows are fed with a feed according to Claim 4.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 11 5697

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION
P,X	EP-A-0 564 704 (AJINOMOTO CO., INC.) * page 2, line 48 - page 3, line 52 * * page 9, line 30 - line 33 * * examples 1-3 * * claims 1-3 *	1,2,4,5	A23K1/16 A23K1/18
X	GB-A-1 193 191 (CHEMOFORMA A.G.) * page 1, line 45 - line 51 * * page 3, line 26 - line 27 * * claims 29,37,38 *	1,2,4,5	
A	EP-A-0 342 111 (GUOMARC'H NUTRITION ANIMALE) * claims 1-4 *	1,2,4,5	
A	SCHWEINEWELT, vol.12, no.1, 1987, DE pages 5 - 8 DIETRICH KAHRS 'TOYOCERIN in der Schweineproduktion' * page 7, column 2, paragraph 1 - page 8, column 3, paragraph 1 * * page 7; table 3 *	1,2,4,5	
A	EP-A-0 416 892 (AJINOMOTO CO., INC.) * page 4, line 23 - line 24 * * claims 1-13 *	1,2	
A	EP-A-0 320 320 (SO.GE.VAL S.A.) * claims 1,3 *	1,2	
A	DATABASE WPI Week 7744, Derwent Publications Ltd., London, GB; AN 77-78084Y & JP-A-50 126 872 (KANEGAFUCHI CHEM KK) 6 October 1975 * abstract *	3	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A23K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	11 January 1995	Dekeirel, M

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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